Active sites of β -lactamases

The chromosomal β -lactamases of *Pseudomonas aeruginosa* and *Escherichia coli*

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An acyl-enzyme was isolated from certain chromosomal β -lactamases and a penicillin. The penicillin was cloxacillin which, although it is a substrate for these enzymes, has such a low k_{cat} that it functions as an inhibitor. The enzymes were from the mutant of *Pseudomonas aeruginosa* 18S that produces the β -lactamase constitutively [Flett, Curtis & Richmond (1976) *J. Bacteriol.* **127**, 1585–1586; Berks, Redhead & Abraham (1982) *J. Gen. Microbiol.*, in the press] and from *Escherichia coli* K-12 (the *ampC* β -lactamase) [Boman, Nordström & Normak (1974) *Ann. N.Y. Acad. Sci.* **235**, 569–586]. The acyl-enzymes have been degraded to determine the residue labelled, and the sequence around it. The residue labelled is serine. The sequences around the labelled serine in these two β -lactamases are exceedingly similar. However, the sequences are quite different from those around the active site serine in the β -lactamases previously studied. There is thus more than one class of serine β -lactamases.

β -Lactamases are hydrolytic enzymes that catalyse the cleavage of the four-membered ring in penicillins and cephalosporins. The latter are β -lactam antibiotics, widely used in medicine; they together represent 60% of world antibiotic sales (Bucourt, 1981). The antibacterial action of penicillins and cephalosporins depends on an intact β -lactam ring; the products of β -lactamase action lack antibacterial activity. Resistance to antibiotics is a wide-spread and increasing clinical problem. Bacteria that are resistant to β -lactam antibiotics usually owe their resistance mainly or partly to their production of β -lactamases. Yet until recently little was known about the active sites of these important and efficient enzymes (Waley, 1981). We suggested, on the basis of affinity-labelling, that certain β -lactamases were serine enzymes (Knott-Hunziker *et al.*, 1979). Subsequent work on β -lactamases both from Gram-positive (Cohen & Pratt, 1980; Cartwright & Coulson, 1980) and Gram-negative (Fisher *et al.*, 1981) bacteria has supported this suggestion. These β -lactamases, on the basis of sequence homology, have been grouped together as one structural class, called class A (Ambler, 1980). On

the other hand, the chromosomal β -lactamase from *Escherichia coli* K-12 (referred to as the *ampC* enzyme) differs structurally from the class A enzymes and has been assigned, on this basis, to class C (Jaurin & Grundström, 1981). What the *ampC* enzyme does resemble, in its catalytic properties, is the chromosomal β -lactamase from *Pseudomonas aeruginosa* (Richmond & Sykes, 1973; Sykes & Matthew, 1976). We recently identified a penicilloyl-serine at the active site of this enzyme (Knott-Hunziker *et al.*, 1980). The present work extends the sequence around the labelled serine to 14 residues. Moreover, we identify the site labelled in the acyl-enzyme of the *ampC* β -lactamase, and are thus able to compare the active sites of these two class C β -lactamases, and to contrast them with the active sites of class A β -lactamases. A semisynthetic penicillin, cloxacillin (I), has been used throughout this work.

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Materials and methods

The β -lactamase from *Ps. aeruginosa* and the [^3H]cloxacillin were as described previously (Knott-Hunziker *et al.*, 1980), and the *ampC* β -lactamase was purified as previously described (Jaurin & Grundström, 1981). The [^3H]cloxacillin was prepared by acylation of 6-aminopenicillanic acid labelled with ^3H in its 2 β -methyl group (Usher *et al.*, 1975); the crystalline sodium salt had sp. radioactivity 4.1 $\mu\text{Ci}/\mu\text{mol}$. Trypsin (twice-crystallized) was from Worthington, chymotrypsin was from BDH and thermolysin was from Calbiochem-Behring. The tryptic digest of the labelled *Pseudomonas* enzyme was prepared as described (Knott-Hunziker *et al.*, 1980). Radioactive peptides were detected by fluorography (Laskey & Mills, 1975; O'Sullivan *et al.*, 1979), or by scintillation counting, and radioactivity was measured on a Nuclear-Chicago Unilux IIA scintillation counter, with Unisolve 1 (Koch-Light) as scintillator.

H.p.l.c. was carried out with a Waters Associates apparatus, on a column (25 cm $\times$ 0.46 diam.) filled with Lichrosorb RP-8 (5 μm) (Owens Polyscience, Macclesfield, Cheshire, U.K.). Gradient elution was used, and u.v. detection at 225 nm was performed in a 10 mm path length cell.

Electrophoresis at pH 6.5 was carried out on Whatman 3MM paper in pyridine/acetic acid/water (25:1:225, by vol.) by the method of Katz *et al.* (1959), and the mobility was measured and expressed relative to aspartic acid = -1 (Offord, 1977). Peptides were detected with the ninhydrin reagent of Heilmann *et al.* (1957). *N*-Terminal amino acids were identified as their dansyl derivatives (Gray, 1972) by t.l.c. on 5 cm square polyamide sheets (Hartley, 1970).

Automated Edman degradation was carried out by Dr. Jean Gagnon (Department of Biochemistry, University of Oxford) in a Beckman spinning cup sequencer, the phenylthiohydantoins being identified by h.p.l.c. (Campbell *et al.*, 1981). The 0.3 M-Quadrol program (Hunkapiller & Hood, 1978) was used, and Polybrene (2 mg) was added to the sequencer cup before each sample (Klapper *et al.*, 1978).

Peptides were hydrolysed in 5.7 M-HCl (BDH Aristar grade) containing 0.1% 2-mercaptoethanol, *in vacuo*, at 108°C for 20 h; the hydrolysates were analysed on an LKB Biochrom 4101 analyser, or a Durrum D-500 amino acid analyser.

Results

Isolation of labelled peptides from the *Pseudomonas* β -lactamase

The tryptic digest of the labelled enzyme (68 mg) was dissolved in 5% (v/v) formic acid and frac-

tionated on a column (140 cm $\times$ 0.9 cm diam.) of Sephadex G-25 (superfine grade) with 0.5% formic acid at 4°C. Fractions containing the radioactive peptide (260 nmol) were pooled and freeze-dried. The next step was h.p.l.c. (Fig. 1), carried out on portions containing about 50 nmol of radioactive peptide. Fractions (50–54) containing radioactive peptide were pooled and freeze-dried. Since the relatively non-polar penicilloyl moiety derived from cloxacillin has an appreciable effect on the position of the labelled peptide in h.p.l.c., selective purification should be possible by re-chromatography of the peptide after the penicilloyl group has been split

Fig. 1. H.p.l.c. of tryptic peptides from *Pseudomonas* β -lactamase

Chromatography was carried out with a linear gradient from 50 mM-triethylamine acetate, pH 4.5, in 5% (v/v) propan-1-ol to the same buffer in 36% (v/v) propan-1-ol. The gradient took effect over 25 min, at a flow rate of 1 ml/min, fractions being collected every 0.5 min. Similar runs had shown that no radioactive material was detected in the first 50 fractions. (a) Tryptic digest, radioactive peptides after fractionation on Sephadex G-25. (b) Re-run of pool from (a) after treatment with triethylamine.

off. Hence the radioactive peptide (49 nmol) was treated with 0.45 ml of 7% (v/v) triethylamine for 100 min at 37°C (cf. Waxman & Strominger, 1980). After freeze-drying, the peptide was submitted to h.p.l.c. again, under the same conditions (Fig. 1b), and was detected in fractions 45–47 by paper electrophoresis as an acidic (Fig. 2) spot, ($m = -0.18$), the colour with the cadmium/ninhydrin reagent being characteristically slow to appear. The electrophoretic mobility of the tryptic peptide was known from an earlier experiment which is now described in outline. The radioactive peptide, isolated after paper electrophoresis at pH 6.5 of a tryptic digest, was treated with triethylamine, and the peptide purified by electrophoresis again at pH 6.5. The only acidic ninhydrin-positive product had mobility -0.18 , gave a slow ninhydrin reaction on paper, and had an amino acid composition virtually identical with that shown in Table 1, column 1. The other fractions of Fig. 1(b) did not

Fig. 2. Paper electrophoresis of labelled peptides

Paper electrophoresis at pH 6.5 was carried out for 35 min at 70 V/cm. The panel on the left shows the distances travelled by the dye markers, the faster being Acid Fuchsin (pink) and the slower Xylene Cyanol FF (blue). The centre panel shows the tryptic peptide from the *Pseudomonas* β -lactamase (calc. charge -2 , 14 residues), its products after treatment with 7% (v/v) triethylamine at 37°C, and the thermolysin peptide (calc. charge -1 , three residues) derived from the tryptic peptide. The panel on the right shows the chymotryptic peptide (calc. charge -1 , nine residues) from the *ampC* β -lactamase, its products after treatment with 7% (v/v) triethylamine at 37°C, and the thermolysin peptide (calc. charge -2 , four residues) derived from the chymotryptic peptide. ////, radioactivity; //, reaction with the cadmium/ninhydrin reagent. The fast elongated radioactive spot is the penicilloic acid derived from cloxacillin, cleaved off the peptides under alkaline conditions.

contain acidic ninhydrin-positive compounds and the nature of the material responsible for the u.v. absorption has not been established. The composition of the tryptic peptide is given in Table 1; the amino acid sequence was determined by automated Edman degradation (Fig. 3).

The labelled tryptic peptide (60 nmol) was digested with 0.2 mg of thermolysin in 0.26 ml of 1% NH_4HCO_3 for 50 min at 37°C; the digest was freeze-dried and then fractionated by electrophoresis on Whatman no. 54 paper at pH 6.5 (80 V/cm for 35 min). There was one radioactive spot detected by fluorography, and the peptide isolated had the sequence Ile-Gly-Ser (Knott-Hunziker *et al.*, 1980) (peptide 1, Fig. 3). Three other ninhydrin-positive spots were isolated and their amino acid compositions (expressed as molar proportions) were as follows.

Peptide 2 ($m + 0.62$): valine (0.93), serine (0.90), lysine (1.17).

Peptide 3 ($m - 0.47$, *N*-terminal leucine): leucine (0.88), phenylalanine (0.85), glutamic acid (1.27).

Peptide 4 ($m - 0.42$, *N*-terminal valine): valine (0.80), threonine (1.84), proline (1.18), glutamic acid (1.18).

These results on the thermolysin peptides confirm the sequence of the tryptic peptide deduced by the automated Edman degradation, and show that the penicilloyl moiety is bound to the serine that is in the sequence Ile-Gly-Ser (Fig. 3).

Preparation of the acyl enzyme from *ampC* β -lactamase

There was a pronounced lag when a preincubated solution of cloxacillin and the *ampC* β -lactamase was added to a solution of benzylpenicillin and hydrolysis was measured by the decrease in A_{232} (Waley, 1974). The lag suggested that arrest of the reaction might show much of the enzyme as acyl-enzyme, and this proved to be so. The acyl-enzyme could be isolated by gel filtration under denaturing conditions. Thus the *ampC* β -lactamase

Fig. 3. Sequence of the tryptic peptide from the *Pseudomonas* β -lactamase

The upper line shows the tryptic peptide and the lower line shows the thermolysin peptides derived from it. The symbol $\rightarrow$ refers to the results from the automated Edman degradation, and $\rightarrow$ refers to *N*-terminal amino acids identified as their dansyl derivatives.

Table 1. *Composition of labelled peptides*

Columns (1) and (2) refer to the tryptic peptide from the *Pseudomonas* β -lactamase and cloxacillin. Columns (3) and (4) refer to the chymotryptic peptide and columns (5) and (6) to the thermolysin peptide from the *ampC* β -lactamase and cloxacillin. The values in columns (1), (3) and (5) are from amino acid analysis of hydrolysates (the latter two being the means of duplicate analyses). The values in column (2) are from the automated Edman degradation. The values in columns (4) and (6) are derived from the sequence of Jaurin & Grundström (1981). The Table gives molar ratios, values of <0.25 mol/mol [columns (3) and (5)] or <0.35 mol/mol [column (1)] being omitted. The electrophoretic mobilities (m) refer to pH 6.5 and are relative to aspartic acid ($m = -1$).

Amino acid	Composition (mol/mol of peptide) of:					
	<i>Pseudomonas</i> enzyme		<i>ampC</i> enzyme			
	(1)	(2)	(3)	(4)	(5)	(6)
	From analysis	From sequencer	From analysis	Calc. for 77-85	From analysis	Calc. for 77-80
Threonine	1.78	2	0.90	1		
Serine	1.63	2	2.07	2	1.01	1
Glutamic acid	2.12	2	0.98	1	1.03	1
Proline	0.86	1				
Glycine	1.36	1	1.18	1	1.04	1
Valine	2.09	2	0.98	1		
Isoleucine	1.02	1				
Leucine	1.20	1	0.96	1	0.93	1
Phenylalanine	0.93	1	0.82	1		
Lysine	1.02	1	1.13	1		
m	-0.34*, -0.18†		-0.25*, 0†		-0.64	

* Mobility of labelled peptide.

† Mobility of peptide after removal of the penicilloyl group.

(about 200 nmol in 1 ml of pH 7.4 buffer) was treated with 20 μ l of 100 mM-[3 H]cloxacillin (sp. radioactivity 4.1 μ Ci/ μ mol) for 1 min at 30°C; the buffer was 0.05 M-NaH₂PO₄/0.05 M-Na₂HPO₄ adjusted to pH 7.4. The reaction was stopped by the addition of 1 ml of glacial acetic acid and the mixture was fractionated at 4°C on a column (20 cm $\times$ 1.5 cm diam.) of Sephadex G-25 (superfine grade) with 30% (v/v) acetic acid. Fractions containing protein (detected by A_{280}) were pooled and freeze-dried. The extent of labelling was 0.82 ± 0.02 mol/mol (mean $\pm$ S.E.M., from six experiments). It is possible that this value is less than 1.0 because deacylation is not much slower than acylation.

Isolation of labelled peptides from ampC β -lactamases

Trial digestions of the labelled *ampC* enzyme by trypsin were unpromising: several labelled peptides were formed. In hindsight, this can be ascribed to the Lys-Lys sequence (Fig. 5). Only one labelled peptide, however, was detected in the chymotryptic digest which (again in hindsight) arises by cleavage after the phenylalanine residues (76 and 85, Fig. 5). The labelled protein (140 nmol) was treated with 60 mg of urea (BDH Aristar grade) and 0.15 ml of 1% NH₄HCO₃ and then was precipitated in a flocculent form with 1.1 ml of 1% NH₄HCO₃.

Chymotrypsin (0.11 ml of a 1% solution in 1 mM-HCl) was added and the mixture was incubated with swirling for 2 h at 37°C. Nearly all of the material, and 85% of the radioactivity, was in the soluble fraction, which was freeze-dried before chromatography on DEAE-Sephacel at 4°C. The column (15 cm $\times$ 0.9 cm diam.) had been equilibrated with pH 6 buffer (20 mM-pyridine brought to pH 6 with acetic acid) and was eluted with a linear gradient (75 ml + 75 ml) from the above buffer to 100 mM-pyridine brought to pH 4.5 with acetic acid. Fractions containing radioactive peptide (about 70 nmol) were pooled and freeze-dried. The peptide ($m = -0.25$, Fig. 2) was further fractionated by reversed-phase h.p.l.c. at pH 4.5 (Fig. 4). The fractions (49-57) containing radioactive peptide were pooled and freeze-dried, and the peptide (28 nmol) was treated with 0.2 ml of 7% (v/v) triethylamine for 1.25 h at 37°C to remove the penicilloyl group. The solution was freeze-dried and the peptide was fractionated by h.p.l.c. under the same conditions (Fig. 4b). The peptide was detected (in fractions 39 and 40) by paper electrophoresis, and was now neutral (Fig. 2). None of the other fractions contained detectable ninhydrin-positive material. The composition of the peptide is given in Table 1, and is there compared with the composition expected for the nonapeptide comprising residues 77-85.

Fig. 4. H.p.l.c. of chymotryptic peptides from *ampC* β -lactamases

Chromatography was carried out at 28°C with a linear gradient from 50mM-triethylamine acetate, pH 4.5, in 5% (v/v) propan-1-ol, to the same buffer in 60% (v/v) propan-1-ol; the gradient took effect over 30min, and the flow rate was 0.8ml/min, fractions being collected every 0.5min. (a) Chymotryptic digest after fractionation on Sephadex DEAE-Sephacel. (b) Re-run of pool from (a) after treatment with triethylamine.

A smaller radioactive peptide was isolated as follows. After the first fractionation by h.p.l.c., the peptide (30nmol) in 0.2ml of 1% NH_4HCO_3 was treated with 20 μl of 0.4% thermolysin in 1% NH_4HCO_3 containing 1.3mM- CaCl_2 for 45min at 37°C. The solution was freeze-dried, and the thermolysin peptide (15nmol) was isolated by h.p.l.c. carried out under the conditions described above, except that 50mM-triethylamine formate buffer (pH 3.5) replaced the triethylamine acetate buffer. The peptide was homogeneous as judged by paper electrophoresis, and the anodic mobility had increased from 0.25 to 0.64 (Fig. 2). The composition of the peptide is compared in Table 1 with that calculated for the tetrapeptide comprising residues 77–80. This peptide, which contained about one molar proportion of ^3H , had *N*-terminal glutamic acid; the chymotryptic peptide isolated after treatment with triethylamine also had *N*-terminal glutamic acid, together with some contaminating

glycine. The values of the electrophoretic mobilities (Table 1 and Fig. 2) (Offord, 1977) show that the glutamic acid residue in these peptides has a free side chain carboxyl group. Serine is the only other amino acid in the tetrapeptide with a reactive side chain. Thus the penicilloyl moiety derived from cloxacillin must be assigned to the serine residue, serine-80 in the sequence of Jaurin & Grundström (1981).

Discussion

Purification of peptides

Few with experience regard the purification of peptides as an altogether trivial task. Penicilloyl peptides, moreover, tend to be unstable. We have found h.p.l.c. to be a welcome addition to the methods available, especially for work with small (e.g. 20nmol, say 20 μg) amounts of peptide. Triethylamine acetate or formate buffers are volatile and relatively transparent in the u.v. Peptides that are slowly eluted in reversed phase h.p.l.c. require the use of propan-1-ol, as stressed by Mahoney & Hermodson (1980). Final purification of the longer peptides was only achieved by a 'diagonal' procedure (Hartley, 1970); after h.p.l.c., the labelled peptide was treated with base to liberate the corresponding unlabelled peptide and h.p.l.c. was performed again.

Comparison of sequences

The sequence of the tryptic peptide from the *Pseudomonas* enzyme may be directly aligned with the sequence of the *ampC* enzyme (Jaurin & Grundström, 1981) on the basis of the serine residue that is labelled by the penicillin (Fig. 5). When this is done, the similarity between the two sequences is clear. Eleven out of the 14 residues are identical. All three mismatches can be explained by a single base change in the codons. This is a striking extent of resemblance. Comparison of the sequences of class A β -lactamases, listed by Ambler (1980), in the region around the active site serine shows that there are five residues in the same positions, shown in the consensus sequence in Fig. 5. Of these five, three (phenylalanine, serine, lysine) occupy corresponding positions in the sequences of the class C enzymes (when the active site serine residues are aligned). It is an obvious possibility that the lysine residue may play a role in catalysis, perhaps donating a proton to the nitrogen atom of the β -lactam. A comparably-placed lysine residue is present in several, but not all, DD-carboxypeptidases (Waxman & Strominger, 1980; Duez *et al.*, 1981).

Class C and class A β -lactamases differ so much in their sequences that they appear to be unrelated in an evolutionary sense (Jaurin & Grundström, 1981). Yet they share an active site serine and function by

Fig. 5. Active site sequences of β -lactamases

The sequence of the *ampC* β -lactamase, which is from Jaurin & Grundström (1981), is shown on the top line, and beneath it are shown the two peptides characterized in the present work: these identify serine-80 as the residue labelled. The next sequence shows the tryptic peptide from the *Pseudomonas* enzyme (present work), and beneath it the thermolysin peptide obtained previously (Knott-Hunziker *et al.*, 1980). The consensus sequence of the class A enzymes listed by Ambler (1980) shows only the amino acid residues (in the region 55–75) common to the four sequenced members.

an acyl-enzyme mechanism. This is reminiscent of the serine proteinases chymotrypsin and subtilisin (Kraut *et al.*, 1972). Perhaps, when more is known of the active site environments in class A and class C β -lactamases, we shall be provided with another example of convergent evolution.

Cloxacillin as substrate and inhibitor

Cloxacillin is well-known as an inhibitor of the class C β -lactamases being studied here (Sabath *et al.*, 1965; Richmond & Sykes, 1973). Now most β -lactams that are inhibitors of β -lactamases are substrates, with a low K_m , numerically equal to the measured K_i (Kiener & Waley, 1978), and a very low k_{cat} . β -Lactam inhibitors of β -lactamases strikingly resemble protein inhibitors of proteinases: both are substrates with low K_m and low k_{cat} , that form acyl-enzymes (Laskowski & Kato, 1980). The combination of a low K_m and a low k_{cat} , leads to a moderate k_{cat}/K_m of the order of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cloxacillin and the *Pseudomonas* enzyme (Knott-Hunziker *et al.*, 1980). The specificity of an enzyme is best measured by k_{cat}/K_m (Brot & Bender, 1969; Fersht, 1977), and on this basis cloxacillin is not such a bad substrate. With high enough concentrations of enzyme, cloxacillin functions as a substrate for class C β -lactamases, and does not inactivate them. There is thus no reason to think that evidence about the structure of the active site of class C β -lactamases arrived at by the use of cloxacillin is misleading. Nevertheless, kinetic evi-

dence about the role of the acyl-enzyme in class C β -lactamases is the next requirement.

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